

# Redox-regulation of haemostasis in hypoxic exercising humans; a randomised double-blind placebo-controlled antioxidant study

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## KEY POINTS

- In-vitro evidence has identified that coagulation is activated by increased oxidative stress, though the link and underlying mechanism in humans has yet to be established.
- We conducted the first randomised controlled trial in healthy participants to examine if oral antioxidant prophylaxis alters the haemostatic responses to hypoxia and exercise given their synergistic capacity to promote free radical formation.
- Systemic free radical formation was shown to increase during hypoxia and was further compounded by exercise; responses that were attenuated by antioxidant prophylaxis.
- In contrast, antioxidant prophylaxis increased thrombin generation at rest in normoxia, and was restored but only in the face of prevailing oxidation.
- Collectively, these findings suggest that human free radical formation is an adaptive phenomenon that serves to maintain vascular haemostasis.

## Abstract

In-vitro evidence suggests that blood coagulation is activated by increased oxidative stress although the link and underlying mechanism in humans has yet to be established. We conducted the first randomised controlled trial to examine if oral antioxidant prophylaxis alters the haemostatic responses to hypoxia and exercise. Healthy males were randomly assigned double-blind to either an antioxidant ( $n = 20$ ) or placebo group ( $n = 16$ ). The antioxidant group ingested 2 capsules/day that each contained 500 mg of *L*-ascorbic acid and 450 international units (IU) of *dl*- $\alpha$ -tocopherol acetate for eight weeks. The placebo group ingested capsules of identical external appearance, taste, and smell (cellulose). Both groups were subsequently exposed to acute hypoxia and maximal physical exercise with venous blood sampled pre-supplementation (normoxia), post-supplementation at rest (normoxia and hypoxia) and following maximal exercise (hypoxia). Systemic free radical formation [electron paramagnetic resonance spectroscopic detection of the ascorbate radical ( $A^{\bullet -}$ )] increased during hypoxia ( $15,152 \pm 1,193$  AU vs.  $14,076 \pm 810$  AU at rest,  $P < 0.05$ ) and was further compounded by exercise ( $16,569 \pm 1,616$  AU vs. rest,  $P < 0.05$ ), responses that were attenuated by antioxidant prophylaxis. In contrast, antioxidant prophylaxis increased thrombin generation as measured by thrombin-antithrombin complex, at rest in normoxia ( $28.7 \pm 6.4$  ug/mL vs.  $4.3 \pm 0.2$  ug/mL pre-intervention,  $P < 0.05$ ) and was restored but only in the face of prevailing oxidation. Collectively, these findings are the first to suggest that human free radical formation likely reflects an adaptive response that serves to maintain vascular haemostasis.

## Introduction

It has been established that physical exercise (Powers & Jackson, 2008) and arterial hypoxaemia (Bailey *et al.*, 2001) independently increase the local and systemic formation of ubisemiquinone, superoxide ( $O_2^{\bullet-}$ ) hydroxyl and nitric oxide free radicals. Human studies in our laboratory have demonstrated that hypoxia promotes free radical-mediated lipid peroxidation subsequent to the metal-catalyzed formation of intermediary lipid-derived oxygen-centered alkoxyl ( $LO^{\bullet}$ ) and carbon-centered alkyl ( $LC^{\bullet}$ ) radicals that is further compounded with the superimposition of acute exercise (Bailey *et al.*, 2004; Bailey *et al.*, 2009b; Bailey *et al.*, 2010; Bailey *et al.*, 2011b; Bailey *et al.*, 2018). Free radical accumulation reduces the endogenous levels of antioxidants, (Bakonyi & Radak, 2004) favouring free radical formation and oxidative stress that when in excess, can result in cellular membrane damage (Lipinski, 2011).

The nutritional antioxidants, ascorbate and  $\alpha$ -tocopherol are thermodynamically well suited to serve as effective aqueous/lipid soluble chain-breaking two electron reductants given their thermodynamic hierarchy and corresponding low one electron reduction potentials [ $E^{\circ}$  of 282 mV for the ascorbate radical ( $A^{\bullet}$ )/ascorbate monoanion ( $AH^{\bullet}$ ) couple (Williams & Yandell, 1982) and 480 mV for the  $\alpha$ -tocoperoxyl radical ( $TO^{\bullet}$ )/ $\alpha$ -tocopherol couple (Simic, 1990)] that are comfortably positioned above most oxidizing species. Furthermore, in combination, they can work synergistically to prevent lipid peroxidation given the thermodynamic capacity of ascorbate to repair ( $\Delta E^{\circ} \approx +200$  mV)  $TO^{\bullet}$  (Tappel, 1968; Packer *et al.*, 1979; Sharma & Buettner, 1993). In support, oral prophylaxis with ascorbate and  $\alpha$ -tocopherol has previously been shown by our group to attenuate systemic  $LO^{\bullet}$ - $LC^{\bullet}$  formation in young participants free of any coagulopathy and improve vascular endothelial function subsequent to increased nitric oxide bioavailability (Richardson *et al.*, 2007; Wray *et al.*, 2009). While the precise scavenging mechanisms remain a matter of debate, ascorbate-

mediated suppression of  $O_2^{\bullet-}$  and hydrogen peroxide formation has been identified as the most thermodynamically cogent candidate though redox-independent mechanisms given that ascorbate can alter expression of  $\alpha$ -ketoglutarate-dependent dioxygenases, hypoxia-inducible factor- $\alpha$  and enzymes involved in histone methylation, also warrant consideration (Cobley *et al.*, 2015).

Inspiratory hypoxia incurred during exposure to terrestrial altitudes ranging from 4,000-6,542 m that approximates to an equivalent to more severe stimulus incorporated in the present study ( $F_{I}O_2$  of 12%  $\approx$  4, 600 m) has been shown to increase thrombin generation (Le Roux *et al.*, 1992; Mannucci *et al.*, 2002). *In-vitro* evidence (Görlach *et al.*, 2000; Görlach, 2004; Herkert *et al.*, 2004) suggests that activation of coagulation is triggered by NADPH oxidase-mediated  $O_2^{\bullet-}$  formation within the blood vessel wall (Görlach *et al.*, 2002). This activates tissue factor *in-vivo* (Herkert & Görlach, 2002), triggering thrombin generation. It has also been shown that hypoxia shifts the endothelial phenotype towards one in which anticoagulant properties are diminished and pro-inflammatory features dominate the endovascular milieu (Ten & Pinsky, 2002; Foley & Conway, 2016). However, these studies have been confined to the *in-vitro* setting thus to what extent haemostasis is subject to redox-regulation in the *in-vivo* human remains to be established.

Thus for the first time, we conducted a randomised, double-blind, placebo-controlled antioxidant trial in young healthy participants that incorporated acute maximal exercise combined with inspiratory hypoxia as established “oxidative stressors”. We hypothesised that based on *in-vitro* findings, hypoxia-induced systemic free-radical formation would be associated with activated coagulation and further compounded with the superimposition of acute exercise subsequent to more pronounced arterial hypoxaemia. We further hypothesised that these responses would be attenuated following antioxidant prophylaxis using a

combination of aqueous/lipid soluble chain-breaking antioxidants with an established capacity to attenuate systemic free radical-mediated lipid peroxidation.

## **Methodology**

### **Ethical Approval**

The study was approved by the University of South Wales Human Research Ethics Committee (UK Ref: LF#27012011). All procedures were carried out in accordance with the Declaration of Helsinki of the World Medical Association (Williams, 2008) except for registration in a database, and written informed consent was obtained from all participants.

### **Participants**

Forty healthy male participants were recruited into the study with a mean  $\pm$  (SEM) age, stature and mass of  $25 \pm 1$  years,  $1.81 \pm 0.01$  m and  $84.8 \pm 2.1$  kg, respectively. Participants were native lowlanders, free of any underlying cardiovascular, pulmonary and cerebrovascular disease and all were non-smokers. They were asked to refrain from consuming any antioxidant supplements or anti-inflammatory medications eight weeks prior to experimentation and follow a low nitrate/nitrite diet for 4 days prior to testing, by avoiding fruits, salads and cured meats (Wang *et al.*, 1997). They were also asked to refrain from caffeine and physical activity for 2 days prior to the study.

### **Design**

The study adopted a randomised, double blind, placebo-controlled antioxidant trial and consisted of 3 phases incorporating nutritional (antioxidant/placebo) and experimental (normoxia/hypoxia/exercise) interventions illustrated in Figure 1 and outlined in detail below:

## Nutritional Interventions

1. Pre-Supplementation, 2. Supplementation and 3. Post-Supplementation.

During phase 1, participants were tested under resting conditions and randomly assigned to a group. During phase 2, they consumed a daily dose of antioxidants or placebo control and in phase 3 they were exposed to inspiratory hypoxia and physical exercise.

***Pre-Supplementation (Phase 1):*** For baseline pre-supplementation testing, participants entered the laboratory at 09:00 following a 12-hour over-night fast and rested in a supine position for 30 minutes prior to blood sampling. Intravenous sampling without stasis was carried out via venepuncture from a prominent antecubital vein using an 18-Gauge precision glide needle (BD Diagnostics, Plymouth UK) into vacutainers (BD Diagnostics, Plymouth UK). Following baseline sampling, participants were randomly assigned to either an antioxidant or placebo group.

***Supplementation (Phase 2):*** The antioxidant group consumed 2 capsules/day (during breakfast and evening meals) that each contained 500 mg of *L*-ascorbic acid and 450 international units (IU) of *dl*- $\alpha$ -tocopherol acetate (RPL Ltd, Blackpool, UK) for eight weeks. The placebo group consumed capsules of identical appearance, taste and smell that each contained an equal quantity of plant cellulose extract but of no nutritional value. They returned to the laboratory exactly 8 weeks after their initial visit for phase 3 of the experiment.

***Post-supplementation (Phase 3):*** This phase of the experiment involved baseline, post-supplementation testing which served a dual purpose: 1. Ascertaining the effectiveness of



phase 2 by checking the blood-borne levels of our target antioxidant vitamins 2: Serving as a new baseline before intervention with inspiratory hypoxia and physical exercise.

## Experimental Interventions

### *Resting measurements in normoxia and hypoxia*

During the experimental day, water was available *ad libitum* and participants were fed a protein shake (1.25% cocoa powder, 0.16% chocolate flavouring, 0.1% aspartame and 98.49% pure whey isolate) made with water (700 mL water: 50g powder) after 3hrs. Participants were asked to wake no later than 07:00 on their day of testing and to immediately consume their morning dose of supplement or placebo. Upon return to the laboratory at 09:30, the participants were once again venepunctured, and baseline sampling was carried out as in Phase 1. After baseline measurements, they were fitted with a pulse oximeter (Nonin Onyx II 9550, Nonin, MN, USA) on their right index finger (Basaranoglu *et al.*, 2015) and exposed to normobaric hypoxia ( $F_I O_2 = 12\%$ ) until completion of the experiment.

Hypoxia was induced using an environmental chamber (Weiss Technik, Ebbw Vale, UK), with controlled temperature (21°C) and relative humidity (50%). The passive exposure period lasted 6 hours, during which time participants were asked to remain on a bed in either a supine or seated position. At 3 hours of exposure time, participants were given their normal dose of either intervention or placebo with water to ensure adequate circulating levels of target vitamins.

### *Exercise*

Following passive exposure to hypoxia, participants were asked to undertake a standardised incremental exercise challenge to volitional exhaustion on an electromagnetically braked

cycle ergometer (Lode Corvial, Lode, Groningen, Netherlands) in hypoxia. The test consisted of maintaining a cadence of 70 revolutions per minute (RPM) on the ergometer, with power output increasing by 35 watts (W) at the end of each minute of exercise. The test was complete when the participant fell below 60 RPM for more than 15 seconds or felt they could no longer continue. Expired gases were collected via an online metabolic cart (Medical Graphics UK, Gloucester UK).

### **Metabolic Measurements**

Bloods were centrifuged at 600 *g* at 4°C for 10 minutes. Plasma and serum samples were decanted into cryogenic vials ((Nalgene® Labware, Thermo Fisher Scientific Inc, Waltham, MA, USA) and immediately snap-frozen in liquid nitrogen (N<sub>2</sub>) and stored at -80°C. Samples were left to defrost at 37°C in the dark for 5 min before batch analysis. Unlike a previous publication (Fall *et al.*, 2011), we specifically chose not to correct blood-borne analytes for plasma volume shifts, notably hypoxia/exercise-induced haemoconcentration, given that researchers do not traditionally account for this potential artefact.

### ***Chemicals and reagents***

These were of the highest available purity from Sigma-Aldrich® (UK).

### ***Haemostasis***

Plasma activated partial thromboplastin time (aPTT), prothrombin time (PT), fibrinogen (FB) and D-dimer (DD) were measured using an ACL Futura Plus, automated coagulometer (Instrumentation Laboratory, Cheshire UK). Blood was collected at each sampling point into sodium citrate Vacutainers (BD Diagnostics, Plymouth UK), spun and separated as described in Phase 1 and stored at -80°C until analysis. aPTT was measured using the HemosIL APTT-

SP (liquid) assay, FB and PT were measured using a joint assay HemosIL PT-Fibrinogen HS Plus and DD was measured using the HemosIL D-Dimer assay. Thrombin-Antithrombin complex (T-AT) was measured using Enzygnost TAT micro (Siemens Medical, Surrey, UK), a sandwich enzyme immunoassay for the *in-vitro* determination of human T-AT. Prothrombin Fragment 1&2 (PF1+2) was measured using Enzygnost F1+2 (monoclonal) (Siemens Medical, Surrey, UK). F1+2 (monoclonal) is an enzyme immunoassay based on the sandwich principle in microtitre format, and based on monoclonal mouse antibodies for the *in-vitro* determination of human PF1+2. The intra and inter-assay CVs were both <5% for all measurements.

### ***Free radicals***

Electron paramagnetic resonance (EPR) spectroscopy was used to directly measure  $A^{\bullet-}$  as a global biomarker of one electron chemistry in plasma and is compatible with either an increased reactivity of systemic free radicals with ascorbate (see later) and/or change in the rate of its repair (Bailey *et al.*, 2009a; Bain *et al.*, 2018). Exactly 1 mL of EDTA plasma was injected into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Daltonics, Billerica, Massachusetts, USA) housed within a TM110 cavity of an EPR spectrometer operating at X-band (9.87 GHz). Samples were analysed using a modulation frequency of 100 kHz, modulation amplitude of 0.65 gauss (G), microwave power of 10 milliwatts (mW), receiver gain of  $2 \times 10^5$  AU, time constant of 41 ms, magnetic field centre of 3477 G and scan width of  $\pm 50$  G for 3 incremental scans. After identical baseline correction and filtering, each of the spectra were subject to double integration using graphical analysis software (OriginPro V.8.5, OriginLab, Massachusetts, USA). The intra and inter-assay CV were both <5%.

### *Antioxidants*

Plasma was stabilised and deproteinated using 10% metaphosphoric acid and ascorbic acid assayed by fluorimetry based on the condensation of dehydroascorbic acid with 1,2-phenylenediamine (Vuilleumier & Keck, 1989). Concentrations of the lipid soluble antioxidants (LSA) including  $\alpha/\gamma$ -tocopherol,  $\alpha/\beta$ -carotene, retinol, lycopene, zexanthin,  $\beta$ -cryptoxanthin and lutein were determined using an HPLC method (Catignani & Bieri, 1983; Thurnham *et al.*, 1988). The intra and inter-assay CV were both <5%.

### *In-vitro study*

**Concept:** In order to disassociate to what extent  $A^{\bullet-}$  reflected “authentic” changes in systemic free radical formation rather than simply an increase in the circulating concentration of substrate (ascorbate) available for oxidation following supplementation, an in-vitro study was performed to determine (and retrospectively) correct for the relationship between ascorbate and  $A^{\bullet-}$ .

**Approach:** Varying concentrations of ascorbate (0, 25, 50, 100, 250, 500, 750, 1000  $\mu$ M) were added to phosphate buffer solution (50 mM, pH 7.4) that had been treated with chelating resin (Chelex 100) via the batch method and the absence of adventitious catalytic metals confirmed as previously outlined (Buettner, 1988). Changes in  $A^{\bullet-}$  were recorded as outlined above and the corresponding relationship determined between the percent (%) increases in ascorbate and  $A^{\bullet-}$ .

### **Statistical analysis**

Following confirmation of distribution normality using Shapiro–Wilks tests, data were analysed using a two-factor mixed ANOVA incorporating one between (group: antioxidant vs. placebo) and one within (condition: rest normoxia vs. rest hypoxia vs. exercise hypoxia)

subjects factor. Following a significant main effect and interaction, Bonferroni corrected paired samples *t*-tests were employed to make *post hoc* comparisons at each level of the within-subjects factor. Between-group comparisons were assessed using independent samples *t*-tests. Data are presented as mean  $\pm$  standard error of mean (SEM) with significance for all two-tailed tests set at  $P < 0.05$ .

## Results

### Compliance

Two participants withdrew from the study and 2 reported prior to phase 3 that they had not complied with their dosing regimen and were subsequently excluded from the overall analysis. Thus, the final data presented are based on a total of 36 participants. The antioxidant group ( $n = 20$ ) were: aged  $25 \pm 1$  years, of stature  $179 \pm 1$  cm, mass  $84 \pm 3$  kg, BMI  $26 \pm 1$  kg/m<sup>2</sup> and the placebo group ( $n = 16$ ) were: aged  $25 \pm 1$  years, of stature  $184 \pm 2$  cm, mass  $85 \pm 3$  kg and BMI  $25 \pm 1$  kg/m<sup>2</sup> ( $P > 0.05$  for all variables).

### In-vitro study

The relationship between ascorbate and  $A^{\bullet-}$  was found to be logarithmic:

$$y (\% \text{ change in } A^{\bullet-}) = 138.37 \ln (x, \% \text{ change in ascorbate}) - 520.79 \quad (r^2 = 0.95)$$

### Pre-supplementation

**Antioxidants:** There were no between group differences ( $P > 0.05$ ) in any of the antioxidants (Table 1).

**Free radicals:** Figure 2 provides typical examples of observed differences in EPR spectral intensities between experimental states with corresponding hydrogen hyperfine coupling constants ( $a^H$ ) of  $\sim 1.76$  G ( $g = 2.0052$ ). There were no between group differences ( $P > 0.05$ ) in  $A^{\bullet-}$  corrected for *de-novo* oxidation of supplemented ascorbate (Figure 3).

**Haemostasis:** There were differences in prothrombin time (PT) between groups (Figure 4D), with the antioxidant group having an elongated PT compared to the placebo group ( $P < 0.05$ ). Other biomarkers of haemostasis showed no differences between groups ( $P > 0.05$ , Figure 4, A-F).

### Post-supplementation

**Antioxidants:** Compared with the placebo, supplementation increased ascorbate (69 vs. 104 ng/mL,  $P < 0.05$ ). There was no difference in  $\alpha$ -tocopherol between groups (38.3 vs. 41.2 mg/mL,  $P > 0.05$ ) but there was an increase in  $\alpha$ -tocopherol pre to post supplementation in the antioxidant group (31.38 vs. 41.22 mg/mL,  $P < 0.05$ ). Retinol increased ( $P < 0.05$ ) in both the antioxidant and placebo groups but there was no difference between groups (1.94 vs. 1.95  $\mu$ mol/L,  $P > 0.05$ ). Supplementation decreased  $\alpha$ -carotene in both groups ( $P < 0.05$ ), but there was no difference between groups (0.04 vs. 0.04  $\mu$ mol/L  $P > 0.05$ ). Other antioxidants were not affected by 8 weeks of supplementation (Table 1).

**Free radicals:** After correction for the increased circulating bioavailability of the antioxidant ascorbate,  $A^{\bullet-}$  was shown to decrease in the antioxidant group ( $9,300 \pm 422$  vs.  $14,076 \pm 810$  AU,  $P < 0.05$ ). The decrease in  $A^{\bullet-}$  was different compared to pre-supplementation ( $15,190 \pm 670$  AU vs.  $9,300 \pm 422$  AU,  $P < 0.05$ ) whereas the placebo control group showed no changes relative to pre-supplementation ( $13,586 \pm 854$  vs.  $14,076 \pm 810$  AU,  $P > 0.05$ , Figure 3).

**Haemostasis:** After 8 weeks of intervention, plasma thrombin-antithrombin complex (T-AT) increased in both groups ( $P < 0.05$ ), but more so in the antioxidant group compared with placebo (11.8 vs. 28.7  $\mu$ g/mL,  $P < 0.05$  Figure 4, A). Prothrombin fragments 1+2 (PF1+2) also increased ( $P < 0.05$ ) post-intervention (Figure 4, B); the antioxidant group had

significantly greater concentrations of PF1+2 post-intervention than the placebo group (126 vs. 198 pmol/L,  $P < 0.05$ ) and compared to pre-intervention, where the placebo group did not change ( $P > 0.05$ ). PT elongated in both groups post-intervention ( $P < 0.05$ ) and the antioxidant group remained elongated compared to placebo ( $P < 0.05$ , Figure 4, D). There were no differences in other markers of haemostasis post-intervention.

## Hypoxia

**Cardiorespiratory measurements:** There were no differences in heart rate or blood oxygen saturation between supplementation groups. After 6 hours exposure to hypoxia, resting heart rate increased in both the antioxidant and placebo groups [ $66 \pm 2$  beats per minute (b/min) vs.  $77 \pm 2$  b/min,  $P < 0.05$ ]. After hypoxic exposure, arterial oxyhaemoglobin saturation ( $S_aO_2$ ) decreased as expected in both groups ( $98 \pm 1$  vs.  $82 \pm 1$  %,  $P < 0.05$ ).

**Antioxidants:** Ascorbate did not increase following exposure to hypoxia vs. normoxia post-supplementation in either group. However, the antioxidant group had significantly higher levels of ascorbate than the placebo group (114 vs. 70 ng/mL,  $P < 0.05$ , Table 2). None of the LSA were affected by 6 hours of hypoxia and there were no differences either within or between groups.

**Free radicals:**  $A^{\bullet-}$  was lower in the antioxidant compared with the placebo group ( $10,583 \pm 443$  AU vs.  $15,152 \pm 1,193$  AU,  $P < 0.05$ ). The increase in  $A^{\bullet-}$  in the antioxidant group compared with normoxia, post-supplementation was not significant ( $9,300 \pm 422$  AU vs.  $10,583 \pm 443$  AU,  $P > 0.05$ ). Hypoxia increased  $A^{\bullet-}$  in the placebo group ( $14,076 \pm 810$  AU vs.  $15,152 \pm 1,193$  AU,  $P < 0.05$ , Figure 2).

**Haemostasis:** Plasma T-AT did not change during hypoxia in the placebo or antioxidant group. However, the difference between the groups following 8 weeks of intervention was

eliminated (Figure 4, A). The Plasma levels of PF1+2 did not alter after exposure to hypoxia, but as with T-AT, the difference between groups following intervention was returned to zero, following exposure to hypoxia (Figure 3, B). Plasma aPTT was not altered by hypoxia in either group (Figure 4, C), PT did not alter through any time point, but as with T-AT and PF1+2, post-intervention differences in PT were again eliminated by hypoxia. Plasma fibrinogen concentrations were unchanged by hypoxia (Figure 4, E) and there were no differences between the intervention groups. D-dimer, the biomarker of blood fibrinolysis also remained unaltered by this stimulus and between groups (Figure 4, F).

## Exercise

**Cardiorespiratory measurements:** Immediately following exercise,  $S_aO_2$  remained lower compared to normoxia ( $98 \pm 1\%$  vs.  $86 \pm 6\%$ ,  $P < 0.05$ ). Exercise increased heart rate vs. normoxia and vs. resting hypoxia ( $66 \pm 2$  b/min vs.  $77 \pm 2$  b/min vs.  $115 \pm 3$  b/min,  $P < 0.05$ ).

**Antioxidants:** As with hypoxia, there were no changes in antioxidants following maximal exercise. Ascorbate remained unchanged compared to normoxia, post-supplementation and in resting exposure to hypoxia, but remained elevated ( $P < 0.05$ ) compared to placebo (Table 2).

**Free radicals:** Normalised  $A^{\bullet-}$  remained lower in the antioxidant compared to the placebo group ( $10,984 \pm 529$  AU vs.  $16,569 \pm 1,618$  AU,  $P < 0.05$ , Figure 3). In the antioxidant group, normalised  $A^{\bullet-}$  increased vs. normoxia, post-supplementation ( $10,984 \pm 529$  AU vs.  $9,300 \pm 529$  AU,  $P < 0.05$ ) and placebo group experienced a similar increase ( $16,569 \pm 1,616$  AU vs.  $15,993 \pm 1,193$  AU,  $P < 0.05$ ).



**Haemostasis:** Exercise increased T-AT in the placebo group ( $P < 0.05$ ), but not in the antioxidant group (Figure 4, A). While PF1+2 did not change following exercise (Figure 4, B) aPTT shortened in both groups ( $P < 0.05$ , Figure 4, C). PT, fibrinogen and D-dimer were unaltered by exercise (Figure 4, D-F).

## Discussion

The present study has revealed two primary findings. First, and as anticipated, hypoxia and to a greater extent, exercise were shown to promote systemic free radical formation, responses that were attenuated by antioxidant prophylaxis. In contrast, and indeed contrary to our original hypothesis, antioxidant prophylaxis increased thrombin generation at rest in normoxia, and was restored but only in the face of prevailing oxidation. Collectively, these findings are the first to suggest that human free radical formation likely represents an adaptive phenomenon that serves to maintain vascular haemostasis.

## Supplementation

The combined increases in the systemic concentration of ascorbate and  $\alpha$ -tocopherol confirms that our participants adhered to the supplementation regimen. This was associated with a corresponding reduction in  $A^{\bullet-}$  (corrected for *de-novo* oxidation of supplemented ascorbate) highlighting a genuine reduction in systemic free radical formation. Following supplementation, the increase in both PF1+2 and T-AT implies increased thrombin generation (Chandler and Velan, 2003) and antithrombin activity (Mannucci, 1994). These data suggest that increased thrombin generation was accompanied by a concomitant increase in the enzyme-inhibitor complex. These data also indicate that global coagulation cascade times and fibrinolysis, as measured by D-dimer, remained unaltered. Collectively, these findings challenge the prior research of Wang et al., (2009) who found that pre-treatment

with chain breaking antioxidant vitamins suppressed thrombin generation albeit within a thrombophilic setting.

### ***Hypoxia***

The concentration of ascorbate in human plasma is orders of magnitude greater than any number of free radicals that exhibit  $E^{\circ'} > 282$  mV (i.e. comparatively more oxidised in terms of thermodynamic hierarchy or “pecking order”) associated with the  $A^{\bullet -}/AH^-$  couple (Buettner, 1993). Thus, when these species are generated within the systemic circulation, they have the thermodynamic potential to react endogenously with this terminal small-molecule antioxidant to form the distinctive  $A^{\bullet -}$  doublet  $[(R^{\bullet} + AH^- \rightarrow A^{\bullet -} + R-H)]$  that is readily observable using EPR spectroscopy (Buettner, 1993). Thus, the elevation in  $A^{\bullet -}$  combined with an inverse relationship with  $SaO_2$  provides direct evidence that hypoxaemia increased systemic free radical formation consistent with our previous findings (Bailey et al., 2009).

Hypoxia was also associated with normalisation of the supplementation-mediated increase in thrombin generation, disagreeing with the current literature. For example, chronic obstructive pulmonary disease (COPD) (Donaldson *et al.*, 2010) and obstructive sleep apnoea (Yaggi *et al.*, 2005) predispose patients to stroke and it is currently thought that this is attributable to systemic oxidation (Donaldson *et al.*, 2010). Notwithstanding other, potentially confounding co-morbidities, the increased risk of atherothrombotic events associated with arterial hypoxaemia may thus be linked to free radical-mediated activation of coagulation.

Previous data from our laboratory also suggest that hypoxia alone and in combination with acute exercise, known to compound systemic oxidative stress, activate coagulation by shortening activated partial thromboplastin time (Fall *et al.*, 2011). However, the present data fail to support this. The reduction in PF1+2 and T-AT suggests that in contrast to what we

originally anticipated, free radical formation during hypoxia normalised the previously thrombophilic profiles of the antioxidant group yet failed to affect the placebo group. Therefore, the data suggest that free radical formation decreases (and not increases, as we anticipated) thrombin generation, contrary to the limited findings reported *in-vitro*.

Previously, authors have suggested that the hypoxia-induced increase in thrombin generation was associated with oxidised low density lipoprotein (LDL-OX)-mediated increased assembly of the prothrombinase complex (Rota et al., 1997) and oxidative stress increased the bioactivity of factor VIII by an as of yet, unknown mechanism (Koprivica *et al.*, 2011). The existing literature regarding the thrombogenicity of hypoxia remains conflicting and our findings collectively support those that state that hypoxia alone fails to alter blood coagulation (Andrew *et al.*, 1987; Crosby *et al.*, 2003; Hodkinson *et al.*, 2003; Fall *et al.*, 2011; Fall *et al.*, 2015).

### ***Exercise***

We have consistently shown that exercise in hypoxia increases  $A^{\bullet-}$ ,  $LO^{\bullet}$  and  $LC^{\bullet}$  (Davison *et al.*, 2006; Bailey *et al.*, 2011a; Woodside *et al.*, 2014; Bailey *et al.*, 2018). In the present study, T-AT increased following exercise in hypoxia in the placebo group but remained unchanged in the antioxidant group. The activation of antithrombin and subsequent generation of T-AT can be interpreted as a consequence of thrombin generation (Burns *et al.*, 2003). Collectively, we interpret these findings to reflect exercise-induced activation of coagulation in the placebo group only, and that antioxidant prophylaxis negated this. PF1+2 remained unchanged following the exercise challenge and therefore these data suggest that the activity of factor X (the principal agent in the conversion of prothrombin to thrombin) was likely unaffected by exercise. This leads to the conclusion that T-AT is elevated because of an increase in the activity of antithrombin (Chandler & Velan, 2003) *per se* and not as a

consequence of increased thrombin generation. To the authors' knowledge, this is a novel finding and could possibly explain previous studies that have shown attenuation of coagulation during hypoxic exercise (DeLoughery *et al.*, 2004). The half-life of PF1+2 is  $\approx$  90 min (Mannucci, 1994) and thus it would be interesting to extend the time-course in follow-up studies or incorporate an alternative biomarker such as fibrinopeptide A given its comparatively shorter half-life ( $\approx$ 5 mins) (Mannucci, 1994).

Activated partial thromboplastin time was shortened post-exercise in both groups, reflecting increased activity of the contact factor pathway of coagulation consistent with our previous research (Fall *et al.*, 2011) and PT remained unchanged which suggests the tissue factor pathway was unaltered. This finding corresponds with research suggesting that exercise promotes expression of vWF (Stakiw *et al.*, 2008), leading to an increase in the vWF-FVIII complex, increasing the activity of the intrinsic coagulation pathway. Furthermore, activation of the coagulation cascade coincided with the increase in  $A^{\bullet -}$ . Recent research in animal models also shows that NADPH oxidases generate  $O_2^{\bullet -}$  promoting platelet activation (Delaney *et al.*, 2016).

It is also known that oxidised lipoproteins support the assembly of the Va/Xa (prothrombinase) complex (Rota *et al.*, 1998) and as such increase thrombin generation as indeed our findings corroborate. Furthermore, studies in patients with antiphospholipid syndrome, which promotes a hypercoagulable state have demonstrated that coagulation is activated by oxidative stress via over-expression of monocyte tissue factor (Ferro *et al.*, 2003). Without the analysis of PF1+2 and T-AT, these data would suggest increased coagulation activation and support the hypoxic findings of Wang and colleagues (2009). Interestingly, the conversion of prothrombin to thrombin is controlled by both pathways and suggests that despite the shortening of aPTT, that there is an inhibitory mechanism that free radicals exert over coagulation and this lies in the activation of antithrombin *per se*.

In contrast, fibrinolysis as measured by D-dimer, remained unaltered throughout the course of the study. This is of interest because acute exercise and exercise training has been shown to increase fibrinolysis in healthy men and in patients with vascular disease (Womack *et al.*, 2003; Killewich *et al.*, 2004). The present data suggest that hypoxia suppresses this, but interestingly Womack and colleagues (2003) argue that the fibrinolytic response to exercise is governed by a participant's baseline "fibrinolytic potential", i.e. the ability to increase fibrinolysis in response to a pro-coagulant stimulus. It is possible that hypoxia is suppressing the fibrinolytic potential of our participants (Mannucci *et al.*, 2002) and that this phenomenon is equally subject to redox regulation *in-vivo*, most likely via a free radical mediated increase in PAI-1 (the principle inhibitor of fibrinolysis) expression.

## Conclusions

The combined application of state-of-the art analytical techniques with an experimental model of hypoxia and exercise known to increase free radical formation, has identified that antioxidant prophylaxis increased resting thrombin generation in normoxia and this was restored only in the face of prevailing oxidation. These findings are the first to suggest that human free radical formation likely represents an adaptive phenomenon that serves to maintain vascular haemostasis, reflecting the hormetic-haemostatic benefits of oxidative stress as we have recently postulated (Bain *et al.*, 2018). Since arterial hypoxaemia in human circulatory disease is associated with oxidative stress and thrombophilia, further research is required to differentiate physiologically adaptive from pathologically maladaptive oxidation.

**Competing interests**

None

**Author contributions**

Intellectual content and study design: LF and DMB. Collection, analysis and interpretation of data: LF, JVB, CJM, DH, KJN, JME, ISY and DMB. Drafting manuscript and graphical representation of data: LF and DMB. Critical evaluation of manuscript: LF, BD and DMB.

All authors approved the submission of the final version submitted

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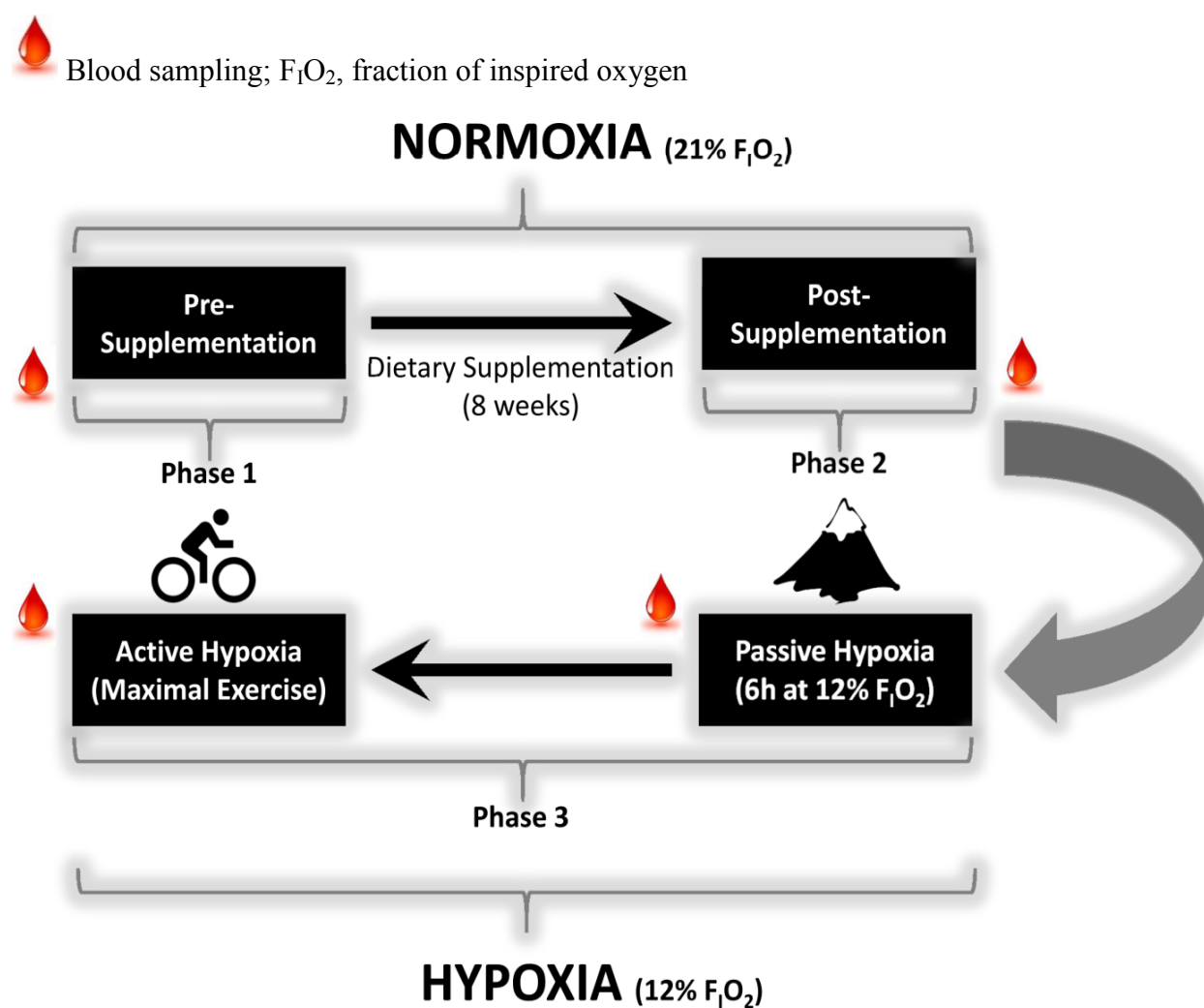
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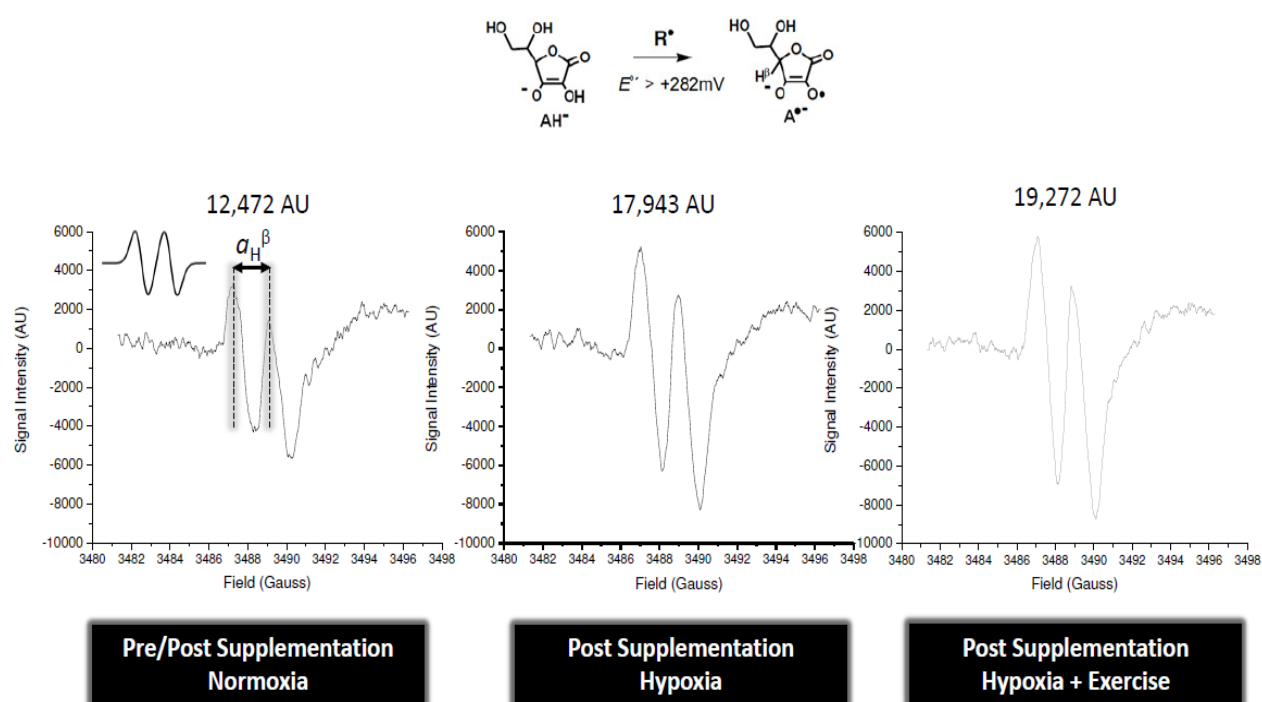
## Legends

**Figure 1: Experimental schema of the randomised double-blind placebo-controlled antioxidant study**



**Figure 2: Typical electron paramagnetic resonance (EPR) spectra of the ascorbate free radical.**

EPR spectra are from the venous circulation of a single participant from the placebo group in normoxia pre/post (placebo) supplementation, following 6 hours passive exposure to hypoxia (12% O<sub>2</sub>) and maximal exercise in hypoxia. All spectra were filtered and scaled identically in arbitrary units (AU). A<sup>•</sup> appears as a (filtered) doublet with a hydrogen hyperfine coupling constant ( $a_H^\beta$ ) of ~1.76 Gauss (see insert of A for simulated spectrum).



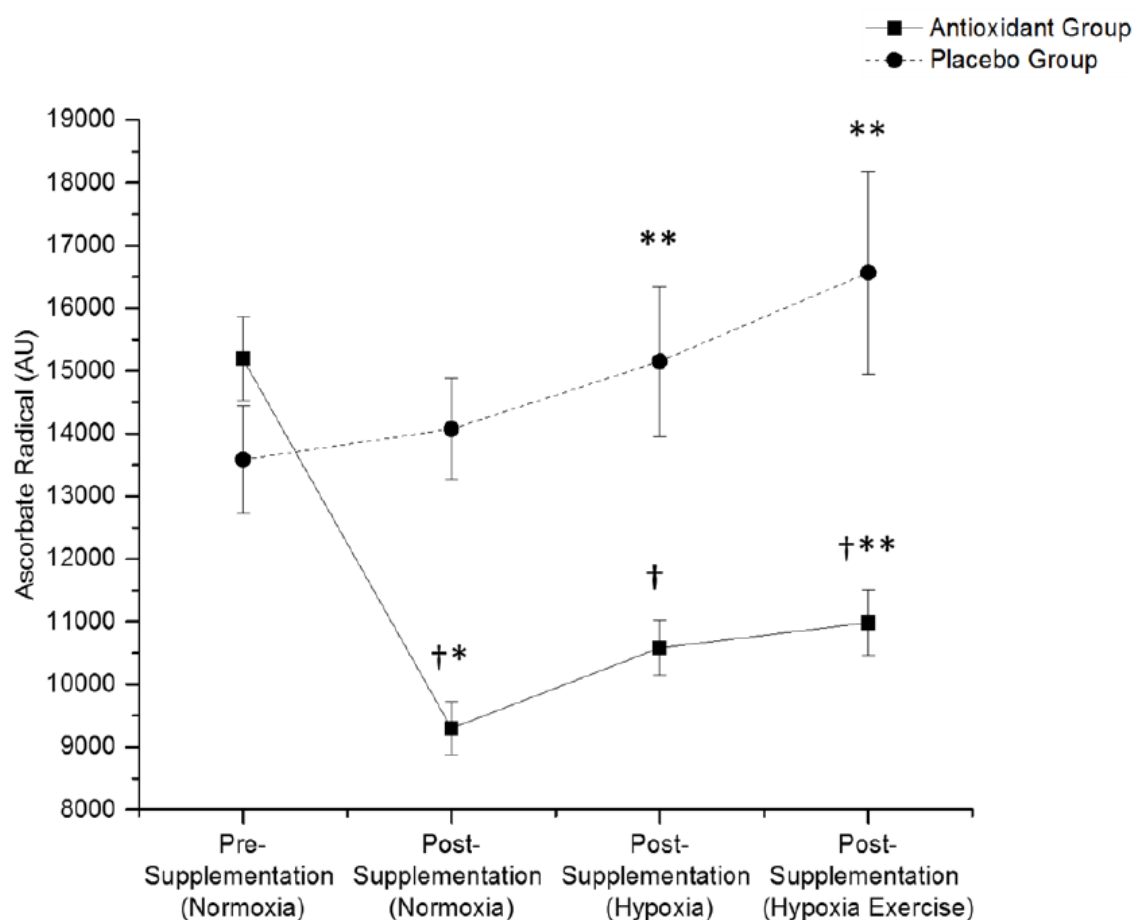
**Figure 3. Effects of supplementation, inspiratory hypoxia and exercise on systemic free radical formation.**

Values are corrected for *de-novo* oxidation of supplemented ascorbate *in-vitro* (see Methods).

Values are mean  $\pm$  SEM error bars. †different vs. placebo group ( $P < 0.05$ ), \* different within



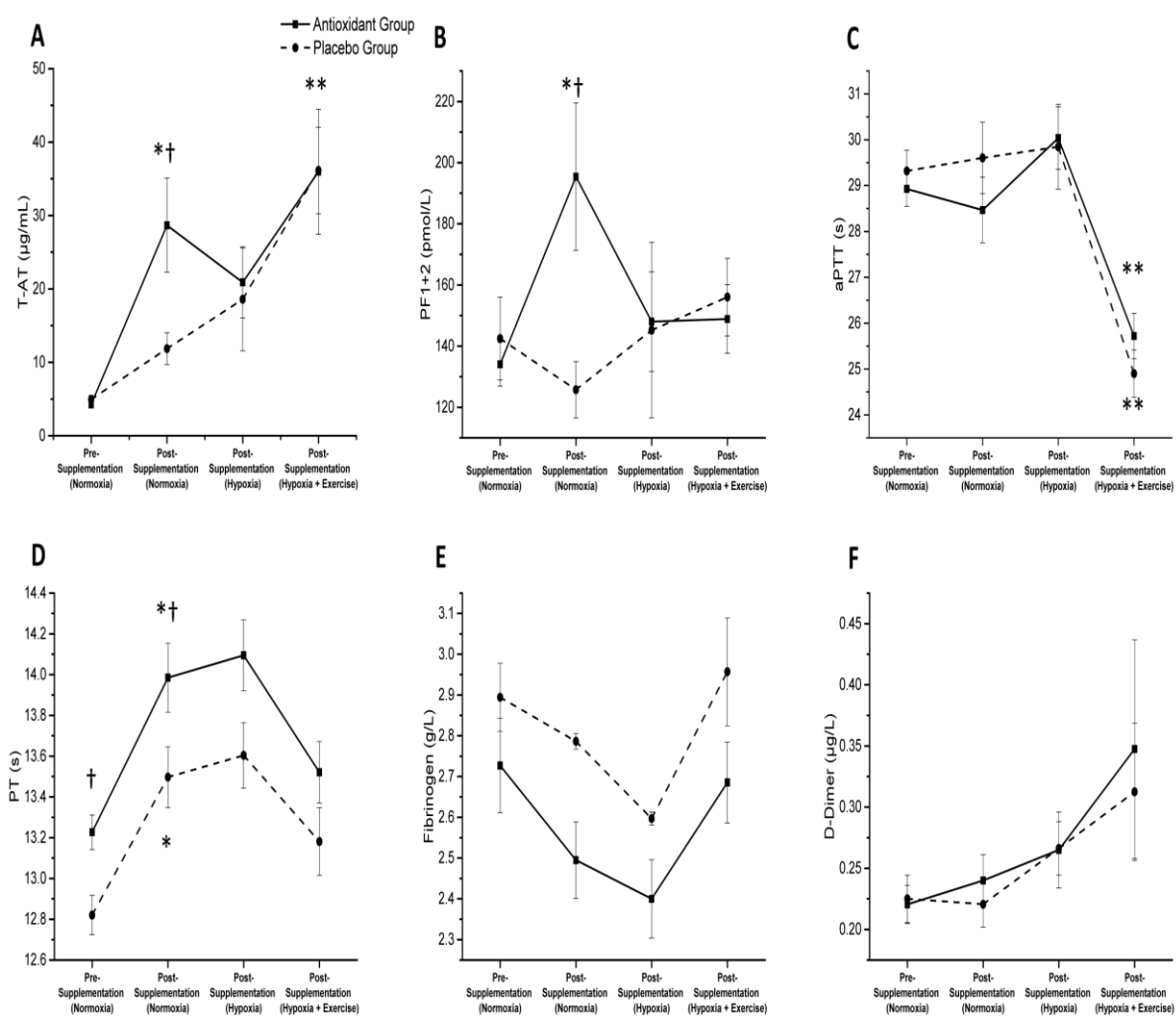
group vs. pre-supplementation normoxia ( $P < 0.05$ ) and \*\*different within group vs. post-supplementation normoxia ( $P < 0.05$ ).



**Figure 4. Effects of supplementation, inspiratory hypoxia and exercise on coagulation and fibrinolysis.**

Values are mean  $\pm$  SEM. T-AT, thrombin-antithrombin complex; PF, prothrombin fragment; aPTT, activated partial thromboplastin time; PT, prothrombin time. \*different within groups

vs. pre-supplementation normoxia ( $P < 0.05$ ), \*\*different within groups vs. post supplementation hypoxia ( $P < 0.05$ ); †different between groups ( $P < 0.05$ ).



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**Table 1. Effects of supplementation on antioxidants**

Group	Placebo group ( <i>n</i> = 16)			Antioxidant group ( <i>n</i> = 20)		
Inspirate	Normoxia			Normoxia		
Phase	Pre	Post	Δ	Pre	Post	Δ
Ascorbate (ng/mL)	74.5 ± 4.0	68.8 ± 4.1	-5.7	70.0 ± 4.1	104.2 ± 11.7*†	34.2
α-tocopherol (mg/mL)	33.3 ± 2.2	38.3 ± 3.1	5.0	31.4 ± 2.8	41.2 ± 3.5*	9.8
γ-tocopherol (mg/mL)	3.4 ± 0.3	1.9 ± 0.5*	1.6	3.3 ± 0.4	1.6 ± 0.2	-1.7
Retinol (μmol/L)	1.11 ± 0.36	1.94 ± 0.92*	0.83	1.18 ± 0.10	1.95 ± 0.17*	0.77
Lutein (μmol/L)	0.10 ± 0.02	0.14 ± 0.02	0.04	0.13 ± 0.02	0.15 ± 0.02	0.02
Zeaxanthin (μmol/L)	0.05 ± 0.01	0.05 ± 0.005	0.00	0.04 ± 0.005	0.04 ± 0.004	0.00
β-cryptoxanthin	0.09 ±	0.08 ±	-0.01	0.09 ±	0.07 ±	-0.02

( $\mu\text{mol/L}$ )	0.01	0.01		0.05	0.03	
$\alpha$ -carotene ( $\mu\text{mol/L}$ )	$0.39 \pm 0.06$	$0.04 \pm 0.08^*$	0.35	$0.38 \pm 0.08$	$0.04 \pm 0.01^*$	-0.34
$\beta$ -carotene ( $\mu\text{mol/L}$ )	$0.80 \pm 0.19$	$0.14 \pm 0.03^*$	0.66	$0.73 \pm 0.16$	$0.12 \pm 0.02^*$	-0.61
Lycopene ( $\mu\text{mol/L}$ )	$3.82 \pm 0.23$	$0.12 \pm 0.01^*$	3.70	$3.38 \pm 0.47$	$0.13 \pm 0.02^*$	-3.25

Values are means  $\pm$  SEM. \*different to pre-supplementation for given group ( $P < 0.05$ ); † different to placebo for given phase of supplementation ( $P < 0.05$ ).

**Table 2. Effects of hypoxia and exercise on antioxidants following supplementation**

Group	Placebo group ( $n = 16$ )			Antioxidant group ( $n = 20$ )		
Inspirate	Normoxia	Hypoxia	Hypoxia	Normoxia	Hypoxia	Hypoxia
State	Rest	Rest	Exercise	Rest	Rest	Exercise
Ascorbate (ng/mL)	$68.8 \pm 4.1$	$70.1 \pm 4.9$	$71.9 \pm 5.1$	$104.2 \pm 11.7$	$113.8 \pm 7.4$	$111.0 \pm 8.5^\dagger$
$\alpha$ -tocopherol (mg/mL)	$38.3 \pm 3.1$	$39.0 \pm 3.2$	$42.8 \pm 3.8$	$41.2 \pm 3.5$	$44.4 \pm 4.4$	$46.0 \pm 3.3$
$\gamma$ -tocopherol (mg/mL)	$1.90 \pm 0.52$	$2.05 \pm 0.45$	$1.57 \pm 0.28$	$1.58 \pm 0.20$	$2.89 \pm 1.0$	$3.05 \pm 0.50$
Retinol ( $\mu\text{mol/L}$ )	$1.94 \pm 0.10$	$1.69 \pm 0.12$	$2.16 \pm 0.08$	$1.95 \pm 0.17$	$1.87 \pm 0.12$	$2.09 \pm 0.27$
Lutein ( $\mu\text{mol/L}$ )	$0.14 \pm 0.02$	$0.13 \pm 0.02$	$0.15 \pm 0.02$	$0.15 \pm 0.02$	$0.14 \pm 0.01$	$0.15 \pm 0.02$
Zeaxanthin ( $\mu\text{mol/L}$ )	$0.05 \pm 0.005$	$0.05 \pm 0.005$	$0.05 \pm 0.005$	$0.04 \pm 0.004$	$0.04 \pm 0.003$	$0.05 \pm 0.005$
$\beta$ - cryptoxanthin	$0.08 \pm 0.01$	$0.07 \pm 0.01$	$0.09 \pm 0.12$	$0.07 \pm 0.03$	$0.06 \pm 0.05$	$0.07 \pm 0.04$

( $\mu\text{mol/L}$ )						
$\alpha$ -carotene ( $\mu\text{mol/L}$ )	$0.04 \pm 0.08$	$0.04 \pm 0.07$	$0.05 \pm 0.07$	$0.04 \pm 0.007$	$0.03 \pm 0.05$	$0.03 \pm 0.003$
$\beta$ -carotene ( $\mu\text{mol/L}$ )	$0.13 \pm 0.03$	$0.12 \pm 0.02$	$0.15 \pm 0.03$	$0.12 \pm 0.02$	$0.09 \pm 0.02$	$0.07 \pm 0.01$
Lycopene ( $\mu\text{mol/L}$ )	$0.12 \pm 0.01$	$0.11 \pm 0.01$	$0.15 \pm 0.02$	$0.13 \pm 0.02$	$0.11 \pm 0.02$	$0.11 \pm 0.02$

Values are means  $\pm$  SEM. †different to placebo group for given inspirate and state ( $P < 0.05$ ).